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Covalent methionylation of *Escherichia coli* methionyl–tRNA synthethase: Identification of the labeled amino acid residues by matrix-assisted laser desorption-ionization mass spectrometry

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Abstract

Methionyl–adenylate, the mixed carboxylic–phosphoric acid anhydride synthesized by methionyl–tRNA synthetase (MetRS) is capable of reacting with this synthetase or other proteins, by forming an isopeptide bond with the ϵ-NH_2 group of lysyl residues. It is proposed that the mechanism for the in vitro methionylation of MetRS might be accounted for by the in situ covalent reaction of methionyl–adenylate with lysine side chains surrounding the active center of the enzyme, as well as by exchange of the label between donor and acceptor proteins. Following the incorporation of 7.0 ± 0.5 mol of methionine per mol of a monomeric truncated methionyl–tRNA synthetase species, the enzymic activities of [^{32}P]PP_i–ATP isotopic exchange and $tRNA^{Met}$ aminoacylation were lowered by 75% and more than 90%, respectively. The addition of $tRNA^{Met}$ protected the enzyme against inactivation and methionine incorporation. Matrix-assisted laser desorption-ionization mass spectrometry designated lysines-114, -132, -142 (or -147), -270, -282, -335, -362, -402, -439, -465, and -547 of truncated methionyl-tRNA synthetase as the target residues for covalent binding of methionine. These lysyl residues are distributed at the surface of the enzyme between three regions [114–150], [270–362], and [402–465], all of which were previously shown to be involved in catalysis or to be located in the binding sites of the three substrates, methionine, ATP, and tRNA.

Keywords: isopeptide bond; MALDI-MS; methionyl–adenylate; methionylated lysyl residues; methionyl–tRNA synthetase; post-translational modification

Post-translational addition of amino acids to proteins is generally believed to occur through the action of aminoacyl–tRNA protein transferases (Soffer, 1971, 1973; Leibowitz & Soffer, 1971a, 1971b; Ingoglia et al., 1983; Zanakis et al., 1984). However, other candidates for covalent reaction with amino acid residues of proteins are the aminoacyl–adenylates. In ribosomal protein translation, aminoacyl–adenylates synthesized by aminoacyl–tRNA synthetases react with the 3′-end of tRNAs. In the case of non-ribosomal biosynthesis of peptide antibiotics, adenylates synthesized at the surface of a multienzyme template react with thiol groups prior to amino acid polymerization (Kleinkauf & Von Döhren, 1990; Pavela-Vrancic et al., 1994).

Because of the high reactivity of their anhydride bond, the synthetase-bound aminoacyl–adenylates are capable to react in vitro with various acceptor molecules such as hydroxylamine or leucinamid to produce aminoacyl–hydroxamate or aminoacyl–leucinamid (Nakajima et al., 1986). In the case of tryptophanyl–tRNA synthetase from beef pancreas, a mixed anhydride between carboxylic groups of the substrate tryptophan and of the synthetase could be shown. It yielded a tryptophanylated enzyme that was further capable of reacting with hydroxylamine (NH_2OH) and of producing hydroxamates of both tryptophan and the enzyme (Kovaleva et al., 1978). Adenylates produced by either aspartyl- or phenylalanyl–tRNA synthetases were shown to modify proteins (Lorber et al., 1982; Kern et al., 1985; Mejdoub et al., 1987; Rapaport et al., 1985). In these cases, the adenylate diffuses outside the catalytic center of the synthetases, prior to the reaction with nucleophilic amino acid residues at the surface of accepting proteins. For example, the product of the reaction involving the ϵ-amino group of a lysine is the formation of an isopeptide bond.

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Abbreviations: HEPES: N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; aminoacyl-tRNA synthetases are abbreviated as a three-letter code of their specific amino acid followed by RS; $MetRS_N$: native dimeric methionyl–tRNA synthetase; M547: the fully active monomeric truncated methionyl–tRNA synthetase.

In this study, we describe the reaction of auto-methionylation of *E. coli* methionyl–tRNA synthetase and the capacity of this enzyme to sustain the modification of accepting proteins. Mass spectrometry analysis enabled us to identify the acylation sites of monomeric truncated methionyl–tRNA synthetase. It is proposed that methionylation occurs by direct reaction of the adenylate with lysines at the vicinity of the active site as well as by exchange of the acylating methionine moiety between a donor and an acceptor protein. In the latter case, diffusion of the reagent in the solvent prior to acylation or bimolecular contact between synthetase-bound adenylate and the acceptor protein may be involved.

Materials and methods

Materials

Homogeneous native dimeric methionyl–tRNA synthetase ($MetRS_N$) was purified from the overproducing strain PAL 1803.5 carrying recombinant plasmid pX1 (Dardel et al., 1984). The fully active monomeric truncated methionyl–tRNA synthetase (M547) was obtained as described (Mellot et al., 1989). Enzyme concentrations were calculated from absorbancy at 280 nm and from the following values of molecular ratio and of optical extinction coefficient: 62.4 K and 1.42 units·mg^{-1}·cm^2 for M547, 152 K, and 1.18 units·mg^{-1}·cm^2 for $MetRS_N$. Initiator $tRNA^{Met}$ from *E. coli* (1.5 nmol of methionine acceptance/A_{260} unit of tRNA) was produced from over-expressing strains of *E. coli* JM101TR grown in 2xTY medium supplemented with 10 μg ampicillin/mL and was purified as already described (Meinnel et al., 1988; Guillon et al., 1992). Inorganic pyrophosphatase from yeast was purchased from Boehringer Mannheim as a suspension in ammonium sulfate at a concentration of 1 mg/mL. N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) and sequence grade $TosPheCH_2Cl$-treated trypsin were from Sigma. [^{14}C]lysine and [^{14}C]methionine were from Dupont de Nemours NEN and exhibited a specific radioactivity of 45–50 mCi/mmol.

Methods

Methionylation of MetRS

Unless stated, methionyl–tRNA synthetase (2 μM) was incubated at 37 °C in 0.1 M HEPES–Na buffer (pH 8.0), containing 2 mM MgATP, 8 mM free $MgCl_2$, 5 μg/mL inorganic pyrophosphatase and 0.2 mM [^{14}C]L-methionine (50 mCi/mmol). Prior to use, the pyrophosphatase samples were dialyzed against 0.1 M HEPES–Na buffer (pH 8.0) to eliminate ammonium sulfate. These conditions will be referred to as the standard conditions for the methionylation reaction. For analytical assays, volumes of the incubation mixtures were 0.2–0.5 mL. At different times, 20–50 μL aliquots were withdrawn and added to 3 ml of 5% trichloroacetic acid (TCA). After addition of 200 μg of bovine serum albumin or of unfractionated yeast RNA, the samples were allowed to stand one hour in an ice bath before filtration on glass fiber filters and counting of the filters in a liquid scintillation counter. When necessary, 3–5 μL aliquots of the incubation mixtures were withdrawn in parallel, diluted at least 100-fold with 20 mM Tris-HCl buffer (pH 7.8) containing 200 μg/mL of bovine serum albumin, 10 mM 2-mercaptoethanol and 0.1 mM EDTA, and assayed for activity in the methionine-dependent [^{32}P]PP_i–ATP exchange and the $tRNA^{Met}$ aminoacylation reactions (Lawrence et al., 1973; Blanquet et al., 1974).

Protection by $tRNA^{fMet}$ or $tRNA^{fMet}ox$

In the protection experiments with periodate-oxidized $tRNA^{fMet}$ (tRNAox), a covalent 95% inactivated M547–tRNAox complex was synthesized as already described (Hountondji et al., 1979). Then the concentration of tRNAox-labeled M547 was set to 2 μM by dilution with 0.1 M HEPES–Na buffer (pH 8.0) containing all the methionylation substrates but [^{14}C]L-methionine, at appropriate concentrations. Control experiments contained M547 and native $tRNA^{fMet}$ instead of tRNAox-labeled M547. Because the M547–tRNAox covalent complex had lost activity, 0.1 μM M547 was added to the corresponding incubation mixture (as well as to the control containing native $tRNA^{fMet}$), to ensure methionyl–adenylate production. Control [^{14}C]L-Met incorporation into 0.1 μM M547 alone was also monitored. Then, the reaction was immediately started by the addition of 0.2 mM [^{14}C]L-methionine (50 mCi/mmol) to the incubation mixtures. At various times, 20 μL aliquots were withdrawn, treated with ribonuclease A (10 μg/mL, 15 min incubation at 37 °C) and precipitated with 5% TCA. In parallel, 20 μL aliquots were withdrawn and directly added to 5% TCA. Control experiments were carried out in the same conditions, without oxidized or native $tRNA^{fMet}$. Ribonuclease treatment of aliquots of the incubation mixtures prior to TCA precipitation destroyed [^{14}C]L-Met–tRNA molecules in which radioactivity would be covalently linked to $tRNA^{fMet}$ or to tRNAox through esterification of the 3′-OH of the ribose and/or through an isopeptide bond with amino groups of the polynucleotides.

Preparation of methionylated peptides

M547 (2.2 μM in 5 mL) was incubated at 37 °C in 0.1 M HEPES–Na buffer (pH 8.0), containing 2 mM ATP, 10 mM $MgCl_2$, 5 μg/mL inorganic pyrophosphatase and 0.2 mM [^{14}C]L-methionine (or non-radioactive L-methionine). After 400 min, when the incorporation had reached a plateau value corresponding to 7.0 ± 0.5 mol of methionine per mol of enzyme, the methionylated M547 was purified by chromatography on a Sephadex G-50 superfine column and digested overnight at 37 °C with $TosPheCH_2Cl$-treated trypsin at a protease to substrate ratio of 1/20 (w/w). The trypsic digest was applied to an Interchrom hypersil C8 column (3.2 × 100 mm, 5-μm particle size) equilibrated with 0.1% TFA in water. The tryptic peptides were then eluted with linear gradients of acetonitrile in 0.1% TFA. Peaks were detected by recording absorbance at 215 and 280 nm, as well as by radioactivity measurements in a liquid scintillation counter. A control M547 sample was digested with trypsin and applied to the Interchrom hypersil C8 column, in the same conditions as above.

Stoichiometry of methionine incorporation

In addition to the stoichiometry measured by TCA precipitation as above, incorporation of methionine into M547 was monitored also as follows. (a) A M547 sample having incorporated [^{14}C]methionine was applied to a Tosohaas TSK 3000 SW column (7.5 i.d. × 30 cm) equilibrated and run with 0.1 M ammonium acetate (pH6.9), at a flow rate of 0.5 mL/mn. The covalent [^{14}C]Met–M547 complex was eluted as a single peak, which was directly quantified by liquid scintillation counting as well as by TCA precipitation. (b) To prepare methionylated M547 for stoichiometry measurement by mass spectrometry, M547 (2.2 μM in 2.5 mL of the standard methionylation mixture) was incubated with 0.2 mM unlabeled methionine. Kinetics of enzyme inactivation was followed in the [^{32}P]PP_i–ATP exchange reaction from 5 μL aliquots of the mixture. In parallel, 0.34 mL aliquots were withdrawn and

applied each on a Interchrom Nucleosil 500 A C4 column (100× 3.2 mm, 7-μm particle size). Separation of the methionylated protein from the smaller substrates was achieved with a 0 to 95% acetonitrile gradient in 0.1% TFA. The Met–M547 covalent complex eluted from the C4 column was submitted to molecular mass measurement by using MALDI mass spectrometry.

Mass spectrum analyses

To analyze the methionylated peptides, samples were prepared by mixing 1.5 μL of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 40% acetonitrile–0.1% TFA) with 1 μL of peptides (5–10 pmol). This mixture was then loaded on a stainless steel sample holder and dried at room temperature. In the case of the analysis of intact Met–M547 covalent complex, 50 mM sinapinic acid was used instead of α-cyano-4-hydroxycinnamic acid.

A VG analytical TOF spec mass spectrometer equipped with a 337 nm laser (MALDI-MS) was used for sample analysis, with a 25 kV acceleration voltage. From 100 to 200 shots were accumulated for each spectrum acquisition in the positive ion mode. Calibration with external standards was obtained with a mixture of bradykinin and insuline in a 1/1 concentration ratio (10 pmol of each species) for peptides analyses, or with 10 pmol of bovine serum albumin for the Met–M547 covalent complex.

Results

Standard conditions for methionyl incorporation

Similarly to yeast aspartyl- and phenylalanyl–tRNA synthetases (Lorber et al., 1982; Kern et al., 1985; Rapaport et al., 1985; Mejdoub et al., 1987), methionyl–tRNA synthetase from *E. coli* undergoes autoaminoacylation with methionine in a time-dependent manner under in vitro amino acid activation conditions (Fig. 1). [^{14}C]methionine did not incorporate into M547 in the absence of either ATP or $MgCl_2$, both of which are known to be necessary for adenylate formation (Fig. 1). The rate and extent of M547 methionylation increased with the [^{14}C]methionine concentration (results not shown), suggesting that the methionyl–adenylate formed by the enzyme itself was the donor species.

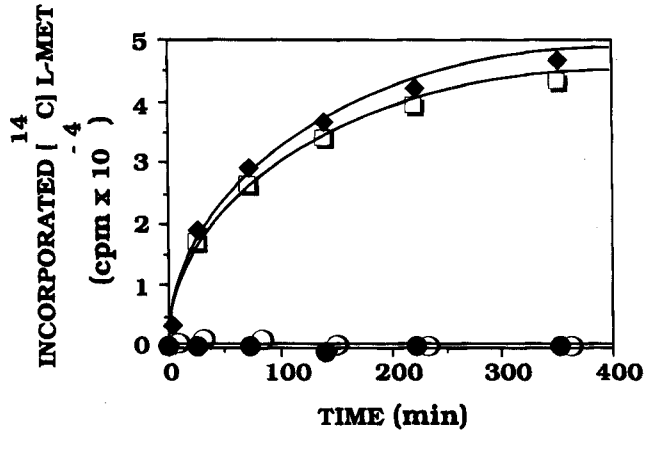

Fig. 1. Time-dependence of methionyl incorporation in M547. The enzyme (3 μM) was incubated at 37 °C in 0.1 M HEPES–Na buffer (pH 8.0) containing 8 mM $MgCl_2$, 10 μg/mL inorganic pyrophosphatase, 0.1 mM [^{14}C]L-methionine (50 mCi/mmol), and MgATP at the concentrations of 1 mM (open squares) or 2 mM (filled diamonds). Incorporation of [^{14}C]L-Met into the enzyme, measured as under Methods, was followed as a function of time. Control experiments were carried out with 2 mM ATP without $MgCl_2$, or with 8 mM $MgCl_2$ without ATP (filled circles). Other control experiments were without ATP and pyrophosphatase, or with ATP and without pyrophosphatase (open circles).

The standard conditions for methionylation of M547 were fixed as follows: 2 μM M547 incubated at 37 °C in 0.1 M HEPES–Na buffer (pH 8.0), containing 2 mM MgATP plus 8 mM free $MgCl_2$, 5 μg/mL inorganic pyrophosphatase and 0.2 mM [^{14}C]L-methionine (45–50 mCi/mmol). Under such conditions, the initial rate of [^{14}C]L-methionine incorporation was 0.8×10^{-3} s^{-1}.

No significant methionine incorporation could be observed in the absence of inorganic pyrophosphatase (Fig. 1). It is likely that pyrophosphatase (5–20 μg/mL) increases the amount of incorporated radioactivity by hydrolyzing PP_i, thereby shifting the equilibrium of the complexes of the enzyme with substrates towards 100% aminoacyl–adenylate formation (Blanquet et al., 1974). In agreement with this explanation, the addition of excess PP_i (20 mM) inhibited methionine incorporation (results not shown).

Methionylation of M547 as a function of methionine concentration

Even though the presence of pyrophosphatase maintained the reaction of adenylate formation out of equilibrium, the rate of methionine incorporation as a function of methionine concentration varied in a hyperbolic manner, with half-effect of methionine at 110 ± 10 μM (Fig. 2). A way to explain such a hyperbolic behavior is to assume that the binding of methionine to its subsite cooperates to dissociate methionyl–adenylate from its site on the synthetase, thereby increasing the ratio of free toward bound acylating reagent. By saturating one subsite of methionyl–adenylate, methionine would decrease the residence time of the latter. In turn, binding of methionine would preclude an adenylate molecule to bind back to a synthetase after having dissociated from its site of origin. Interestingly, a similar behavior of methionine in the dissociation of methionylated $tRNA^{Met}$ was previously proposed with, under similar ionic conditions, half effect of the amino acid corresponding to 50 μM (Jacques & Blanquet, 1977). In this case, free methionine is believed to compete for binding with the methionyl residue esterified at the 3′-end of initiator $tRNA^{Met}$. In truth, in the present study, the effect of methionine in the stimulation of the acylation reaction is likely to be underestimated because of nucleophilic attack of the adenylate by the α-amino group of the amino acid added in excess, as demonstrated by the observation that the initial rate as well as the maximum amount of acylation of M547 by [^{14}C]L-methionine were reduced by twofold in the presence of 0.5 mM D-methionine (results no shown).

Specificity

Once formed by M547, the methionyl–adenylate was capable of reacting with other added proteins, such as bovine serum albumin (BSA), LysRS, or ValRS (results not shown).

The labeling of BSA could be directly evidenced as follows: native dimeric methionyl–tRNA synthetase (2 μM in 0.5 mL of the standard methionylation mixture) was incubated with 5, 10, or 20 μM BSA. At the maximum of [^{14}C]methionine incorporation, the [^{14}C]Met-labeled synthetase was separated from labeled BSA by chromatography on a TSK G 3000 SW column. As shown in Table 1, [^{14}C]L-Met incorporation into BSA increased in parallel

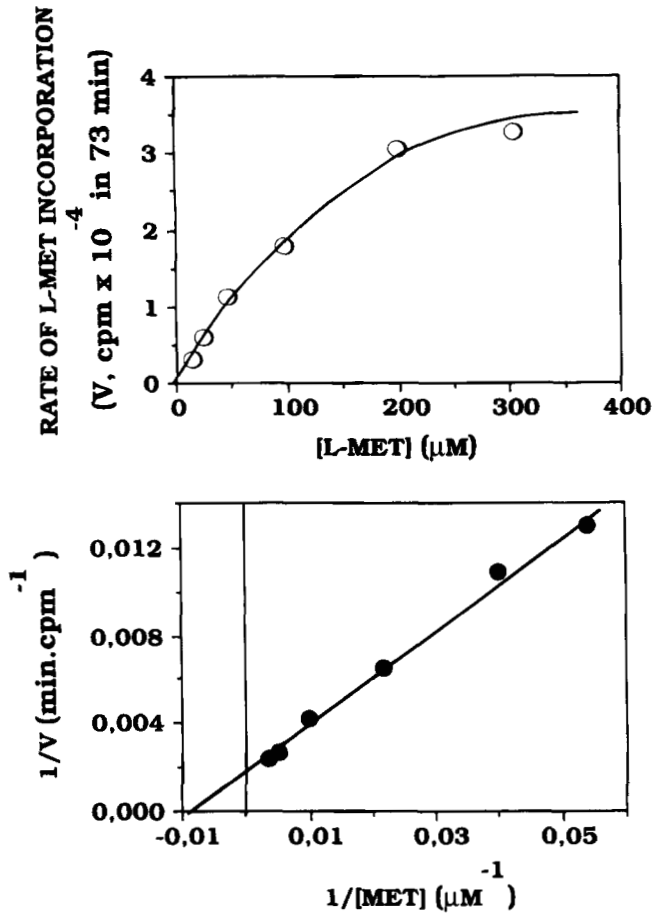

Fig. 2. Variation of the apparent rate of methionine incorporation as a function of methionine concentration. The amount (cpm) of [^{14}C]L-Met incorporated at 73 min incubation time was plotted as a function of free methionine concentration (open circles). A half-effect of methionine at 110 ± 10 μM can be deduced from the double reciprocal plot (filled circles).

with the amount of added BSA, in agreement with the idea that methionyl–adenylate dissociates from the synthetase, prior to the acylation of acceptor proteins, or that BSA is acylated through bimolecular contact with enzyme-bound methionyl–adenylate.

Stoichiometry

The stoichiometry of labeling, as followed by TCA precipitation (see Methods), progressively tended to a plateau value within 5–6 h corresponding to 7.0 ± 0.5 mol of [^{14}C]methionine incorporated per mol of the monomeric M547 species of methionyl–tRNA synthetase (Fig. 3). Measurements were identical if TCA precipitation was performed in the presence of either bovine serum albumin or unfractionated yeast RNA, as carrier. In the case of the native dimeric MetRS, 14.0 ± 1.0 mol of [^{14}C]Met were incorporated by 1 mol of enzyme, under the same conditions (Fig. 3).

[^{14}C]Methionine incorporation was also directly measured by liquid scintillation counting without the step of TCA precipitation. For this experiment, a [^{14}C]Met-labeled M547 sample was separated from free [^{14}C]methionine by chromatography on a TSK G 3000 SW column. Up to 8.0 ± 0.5 mol of [^{14}C]methionine were found associated to 1 mol of the monomeric synthetase. This stoichiometry which is slightly larger than the 7.0 ± 0.5 mol/mol

Table 1. *TSK analysis of native dimeric MetRS methionylated in the presence of bovine serum albunin*[a]

Concentration of BSA (μM)	[^{14}C]L-Met incorporated by native MetRS (cpm × 10^{-4})	[^{14}C]L-Met incorporated by BSA (cpm × 10^{-3})
0	6.3	—
5	6.9	11.6
10	6.5	20.4
20	6.8	34.7

[a]The enzyme (3 μM) was incubated for 400 min in the standard conditions, with none, or with bovine serum albumin (BSA) at a final concentration of 5, 10 or 20 μM. Separation of [^{14}C]Met-labeled proteins was achieved by applying incubation mixtures to a Tosohaas TSK 3000 SW column (7.5 i.d. × 30 cm) equilibrated and run with 0.1 M ammonium acetate (pH 6.9), at a flow rate of 0.5 mL/mn. The covalent [^{14}C]Met-protein complexes were quantified by TCA precipitation.

figure obtained by TCA precipitation, might reflect non-covalently bound adenylate or L-methionine carried by the synthetase under the conditions of the chromatography.

The stoichiometry of methionine incorporation was also measured by MALDI mass spectrometry (results not shown). Methionylated M547 samples obtained after 30 and 150 min of incubation showed mass values increased by 193 and 628 Da, respectively, corresponding to the covalent addition of 1.45 and 4.72 methionyl residues, respectively, in agreement with the values obtained by TCA precipitation of [^{14}C]Met-labeled M547 samples followed in parallel (Fig. 3).

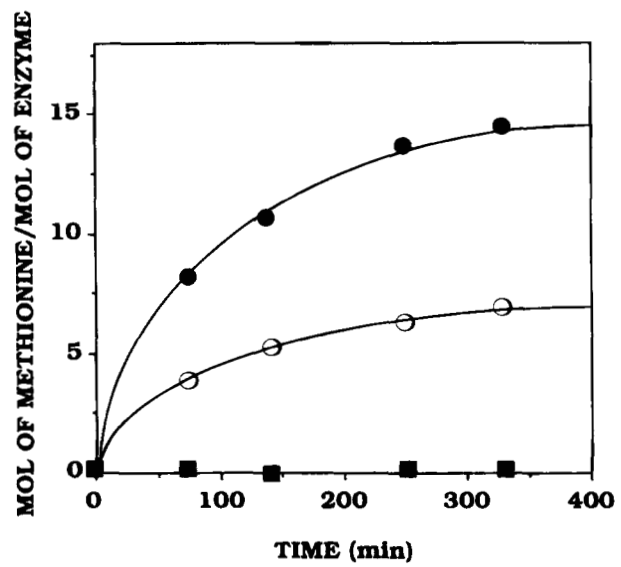

Fig. 3. Stoichiometry of the methionylation reaction. Native dimeric MetRS (2 μM) (filled circles) or the monomeric truncated M547 species (2.2 μM) (open circles) were incubated in the standard conditions described under Methods. The number of mol of [^{14}C]L-methionyl residue incorporated per mol of enzyme was deduced from radioactivity measurement, as described under Methods. The stoichiometries (mol/mol) were plotted as a function of the incubation time. Control experiments with either MetRS species were carried out with 2 mM ATP without $MgCl_2$, or with 8 mM $MgCl_2$ without ATP (filled squares).

The amount of methionine incorporated by M547, as measured by TCA precipitation, increased with the amount of M547 initially present in the incubation mixture (results not shown), in the range 0.3-2.4 μM. In this range, after six hours of incubation at 37 °C, a molar ratio of 7.0 ± 0.5 mol of [^{14}C]methionine/mol M547 was calculated at the maximum of methionine incorporation. This behavior is compatible with a reaction mechanism involving either the dissociation of methionyl–adenylate molecules formed by the enzyme, followed by pre-equilibrium of the reagent with the protein in the mechanism of covalent incorporation, or bimolecular contacts between enzyme molecules in the covalent transfer of methionine from a donor M547 to an acceptor one. However, beyond 2.4 μM M547 in the acylation assay, the amount of incorporated methionine reached plateau values, while the calculated molar ratio of methionine incorporation started to decrease (Fig. 4, and results not shown). For instance, at 10 μM M547, only 4 mol of methionine were bound to one mol enzyme, although [^{14}C]L-Met (100 μM) was not limiting in the experiment. These results suggested that, at high M547 concentrations, methionyl–adenylate, the donor of methionyl residues in the reaction rapidly becomes limiting. The possibility that ATP degradation rapidly occurred at high M547 concentration because of a contaminating adenosine triphosphatase activity was ruled out, however, by the observation that the radioactivity associated with γ[^{33}P]–ATP remained intact after 15 h of incubation with M547 samples at 2, 15, and 25 μM in the absence of L-methionine. Actually, the ATP limitation at high enzyme concentration is likely to be caused by the intrinsic instability of enzyme-bound methionyl–adenylate (Blanquet et al., 1972; Hyafil et al., 1976). Indeed, by increasing the number of adenylate molecules exposed to hydrolysis, an increasing of the number of enzyme molecules accelerates the turnover of ATP.

Effect of methionylation on enzyme activity

As shown in Figure 4, both tRNA^{Met} aminoacylation and methionine-dependent ATP–PP$_i$ exchange activities of M547 decreased in parallel with methionyl incorporation. In the standard conditions, the residual activities of [^{32}P]PP$_i$–ATP exchange and tRNA^{Met} aminoacylation of 2 μM M547 having incorporated 7.0 ± 0.5 mol of [^{14}C]L-Met/mol enzyme were 25 and 6% of that of the intact enzyme, respectively. In the same conditions, 10 μM M547 having incorporated only 4.0 mol of [^{14}C]L-Met/mol enzyme retained 60 and 25% of the initial [^{32}P]PP$_i$–ATP exchange and tRNA^{Met} aminoacylation activities, respectively, indicating that enzyme inactivation varies with the extent of covalent methionine fixation. Finally, it was demonstrated by not shown experiments that the incorporated label could not be further transferred from a covalent [^{14}C]Met–M547 complex to the 3′-end of tRNA^{Met}.

Protection by tRNA against the labeling

The presence of excess tRNA^{fMet} reduced by 94% the rate of [^{14}C]Met incorporation and protected M547 against inactivation (results not shown). An explanation of this behavior is that a bound tRNA macromolecule masks the modification sites along the surface of the synthetase, or unfavors contacts between the acceptor synthetase molecule and the label free or bound. However, one cannot exclude that the turnover of methionyl–adenylate, the donor of methionyl residues, had become limiting because of enzyme saturation by aminoacylated tRNA. The observation that [^{14}C]Met incorporation in a sample composed of M547 (0.1 μM) and BSA

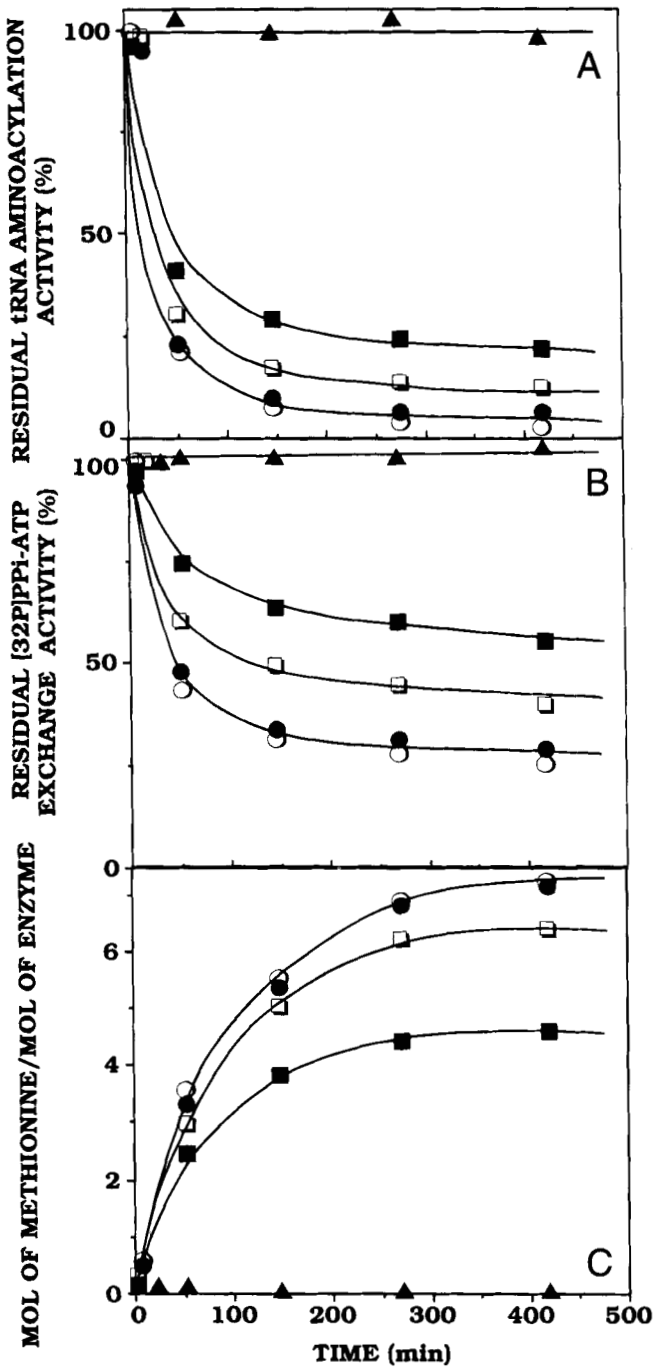

Fig. 4. Effect of methionylation on M547 activity. (**A**) Residual tRNA^{Met} aminoacylation activity (%) of M547, at final concentrations of 1 (open circles), 2 (filled circles), 5 (open squares), or 10 μM (filled squares), is plotted as a function of the incubation time. (**B**) Residual methionine-dependent [^{32}P]PP$_i$–ATP exchange activity (%) of M547, at final concentrations of 1 (open circles), 2 (filled circles), 5 (open squares), or 10 μM (filled squares), is plotted as a function of the incubation time. The residual tRNA^{Met} aminoacylation and methionine-dependent [^{32}P]PP$_i$–ATP exchange activities (filled triangles) were also followed in incubation mixtures with 1, 2, 5, or 10 μM M547, without ATP and pyrophosphatase. (**C**) The number of moles of [^{14}C]L-methionyl residue incorporated per mol of M547 [at final concentrations of 1 (open circles), 2 (filled circles), 5 (open squares), or 10 μM (filled squares)], is plotted as a function of the incubation time. Control experiments (filled triangles) are with 1, 2, 5, or 10 μM M547 and without ATP and pyrophosphatase.

(20 μM) was lower by a factor 2 in the presence of $tRNA^{fMet}$ (0.5 μM) than in its absence (results not shown) is compatible with any of the above explanations.

Effective protection against methionylation of M547 bound to tRNA could be directly evidenced by preparing 2 μM M547 covalently labeled with $tRNA_{ox}^{fMet}$ and by comparing its acylation in the presence or absence of catalytic amounts (0.1 μM) of M547 (the donor protein). As a control, 2 μM of M547 inactivated by 98% with adenosine diphosphopyridoxal (Hountondji et al., 1990a) was also acylated in the presence or absence of 0.1 μM M547. It was verified that, in the same conditions, 0.1 μM M547 sustained measurable acylation of BSA (at 5, 10, or 20 μM). The covalent attachment of oxidized tRNA reduced the amount of [^{14}C]Met incorporation by 99% (results not shown). In contrast, [^{14}C]Met incorporation in adenosine diphosphopyridoxal-labeled M547 was only 40% lower than in the sample containing 2 μM intact enzyme, thereby suggesting that this covalent complex could still accept methionyl residues. Altogether, the above results clearly indicate that inhibition of the methionylation reaction is mostly due to the masking by oxidized tRNA of the modification sites on the synthetase, in agreement with the earlier demonstration that covalently bound $tRNA_{ox}^{fMet}$ fully inactivates M547 and occupies the tRNA site (Hountondji et al., 1985).

[^{14}C]Met-labeled peptides of M547

Automated Edman degradation of [^{14}C]Met-labeled peptides failed to directly identify the lysyl residues, the ϵ-NH_2 groups of which have formed an isopeptide bond with the carboxylate group of methionine. As already discussed by Kern et al. (1985), the reason is that, during the first cycle of Edman degradation, the reagent phenylisothiocyanate reacts both with the α-NH_2 group of the amino terminal residue of the peptide and the free α-NH_2 of the covalently bound methionine. As a consequence, at this cycle, the phenylthiohydantoin (PTH) derivative of [^{14}C]Met was delivered by the sequencer, in parallel with that of the NH_2-terminal residue of the analyzed peptides. Actually, nearly 100% of the covalently bound methionyl residues was released from each peptide at the first cycle of Edman degradation, thus preventing direct identification of a methionylated lysyl residue as a PTH derivative, or of a labeled peptide, in a mixture with other labeled or unlabeled ones.

Similarly, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-MS) could not directly identify the methionylated lysyl residues. However, the latter method was more suitable than Edman degradation for the identification of labeled peptides, because it enabled us to easily identify Met-labeled peptides in a mixture with unlabeled ones, without purification to homogeneity, and without any loss of the Met label. Indeed, MALDI-MS directly measured the mass increase due to the covalent binding of methionyl residues to each peptide. Identification of labeled lysyl residues was based on the observation that all the analyzed Met-labeled peptides systematically contained a single internal lysyl residue that resisted to further trypsin digestion because of the isopeptide bond formed with the carboxylate of the activated methionine. Figure 5 shows a typical mass spectrum of a methionylated peptide of M547. The molecular masses of methionine-labeled peptides are listed in Table 2. Accordingly, methionylatable residues of M547 are lysines-114, -132, -142 (or -147), -270, -282, -335, -362, -402, -439, -465, and -547 (Dardel et al., 1984). Lys-465 appeared to be the predominantly labeled residue (Fig. 5).

Table 2. *Matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) of methionylated peptides of M547*[a]

Labeled peptide sequence	Measured mass (*)	Calculated mass (*)	Labeled residue
^{113}LKENGFIK^{120}	1,088.7	1,083.2	K114
^{123}TISQLYDPEKGMFLPDR^{139}	2,149.1	2,144.3	K132
^{133}GMFLPDRFVKGTCPKCK^{149}	2,065.5	2,061.4	K142 or K147
^{266}NLCDKRGDSVSFDEYWK^{282}	2,201.8	2,196.2	K270
^{272}GDSVSFDEYWKK^{283}	1,598.3	1,594.6	K282
^{333}MSKSR^{337}	742.7	742.1	K335
^{357}YYYTAKLSSR^{366}	1,386.4	1,385.2	K362
^{396}NAGFINKR^{403}	1,053.7	1,053.1	K402
^{436}EFGKAVR^{442}	940.8	939.9	K439
^{454}YVDEQAPWVVAKQEGR^{469}	2,011.5	2,010.1	K465
^{534}IDMRQVEALVEASK^{547}	1,724.1	1,722.8	K547

[a]Calculated and observed masses of methionylated peptides are given for molecular ions in the positive ([M + H]+) ion mode.

The major difficulty in the interpretation of MALDI-MS data is the quantification of the analyzed peptides, following their desorption. Indeed, the rules governing peptides desorption are not yet fully elucidated. As a consequence, apart from major peaks, which undoubtedly correspond to major peptides, the relative height of a peak on a mass spectrum cannot be regarded as a reliable indication of the relative abundance of the corresponding peptide in the mixture.

Discussion

This study establishes that methionyl–tRNA synthetase can be modified by L-methionine from a donor methionyl–adenylate, the mixed carboxylic–phosphoric acid anhydride synthesized by the enzyme itself. It should be noted that supporting evidence for covalent reaction of methionine with methionyl–tRNA synthetase has been already briefly described by Martinis and Schimmel (1992).

Tritiated ATP failed to label the enzyme in the presence of L-methionine (results not shown) indicating that while the adenylate was the donor species, the actual label consisted solely of the amino acid moiety. This was confirmed by the fact that the molecular mass of each methionylated peptide, as measured by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-MS), exactly corresponded to the mass of the peptide having incorporated one [^{14}C]methionyl residue. The bond formed between M547 and methionyl residues was resistant to TCA precipitation as well as to the high energy laser beam during the MALDI-MS analysis of the labeled enzyme or peptides. These observations indicated that methionine was covalently bound to the enzyme. The occurrence of amide (or isopeptidic) bonds was demonstrated by the behavior of methionine-labeled peptides in the N-terminal sequencing by Edman degradation. Indeed, the methionyl residue attached to the [^{14}C]methionylated peptides of M547 was released as PTH-[^{14}C]Met at the first cycle of the Edman degradation.

In the standard conditions, a maximum of 7.0 ± 0.5 mol of [^{14}C]methionine were incorporated per mol of monomeric M547. This stoichiometry of labeling, which is markedly higher than the active stoichiometry of the enzyme (1 mol methionine/mol trun-

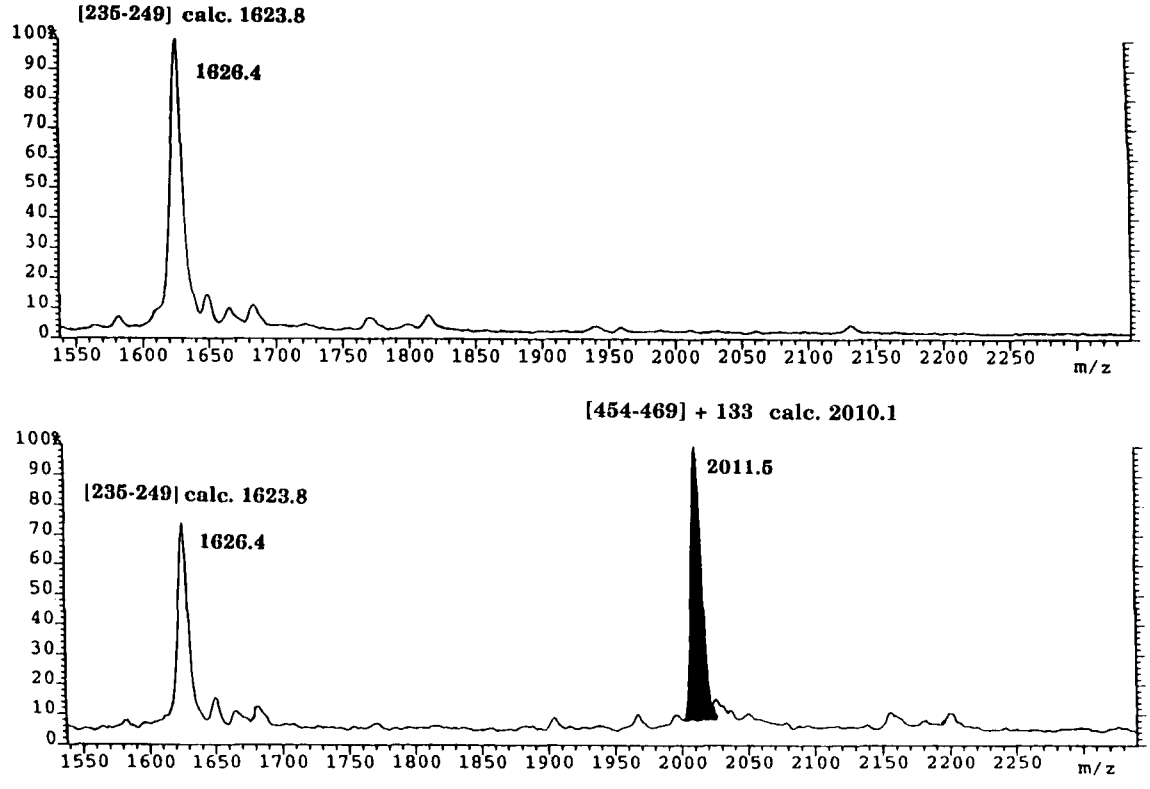

Fig. 5. MALDI mass spectrum of a methionylated peptide of M547. The lower part of the figure shows fragment [454–469] of Met-labeled M547 represented by the black peak (sequence YVDEQAPWVVAKQEGR, calculated average mass with label 2010.1 Da), identified by MALDI-MS in a RPLC fraction containing one major unlabeled peptide. The methionylatable residue is Lys-465. The upper part shows the MALDI-MS analysis of the corresponding RPLC fraction from a tryptic digest of a control sample of intact M547. Only the unlabeled peptide (calculated average mass, 1623.8) was present.

cated enzyme), indicates that labeling occurs at several sites, inside and/or outside the enzyme active site. The finding that 14.0 ± 1.0 mol of [^{14}C]methionine were incorporated per mol of native dimeric MetRS suggests that both MetRS species were methionylated on the same sites.

MALDI-MS designated lysines-114, -132, -142 (or -147), -270, -282, -335, -362, -402, -439, -465, and the carboxyl-terminal lysine-547 of M547 as the targets for covalent binding of the methionyl residue. In the primary structure of M547 (Dardel et al., 1984), apart from the carboxyl-terminal Lys-547, these lysyl residues are clustered between three regions [114–150], [270–362], and [402–465], all of which were previously shown to be involved in catalysis or substrate binding. In particular, fragments [114–150] and [270–362] are partly located in the catalytic crevice of the M547 3D structure (Fig. 6, and Brunie et al., 1990).

Recently, a structure-based multiple sequence alignment of all class I aminoacyl–tRNA synthetases was achieved on the basis of the GlnRS:tRNA^{Gln} co-crystal structure. It revealed that fragment [102–124] of M547 might participate to the binding of the acceptor arm of tRNA^{Met} (Landès et al., 1995). Affinity labeling studies have demonstrated that lysines-142 and -147 are involved, along with Lys-61, Lys-149, Lys-335, and Arg-435, in the binding pocket of the CCA-end of tRNA^{Met} (Fig. 6, and Hountondji et al., 1990b). In addition, fragment 141–161 of M547 was shown to tightly bind one zinc ion per mol of enzyme through a zinc finger-like structure built up with the consensus sequence $CX_2CX_9CX_2C$, at the border of the active site crevice of the M547 3D structure (Fourmy et al., 1993; Landro & Schimmel, 1993). In recent affinity labeling studies, lysine 132 covalently reacted with pyridoxal-5′-phosphate (PLP), thus suggesting that this residue might represent a phosphate-binding locus in the synthetase structure (Kalogerakos et al., 1994).

Lysines 270 and 282 belong to fragment 255–300, which was demonstrated by site-directed mutagenesis studies to be involved in methionine recognition (Fig. 6, and Fourmy et al., 1991; Ghosh et al., 1991). Actually, Arg-233, Val-298, His-301, and Trp-305 participate to the methionine-binding site, while Lys-295 would play a crucial role in the transfer of activated methionine to the 3′-acceptor end of tRNA^{Met} (Fourmy et al., 1991; Ghosh et al., 1991).

Lys-335 of M547 is the second lysyl residue within the KMSKS consensus sequence of class 1 aminoacyl–tRNA synthetases (Hountondji et al., 1985, 1986a, 1986b, 1990a; Eriani et al., 1990; Cusack et al., 1990), and has been shown by affinity labeling studies and by site-directed mutagenesis to correspond to the subsite of the pyrophosphate moiety of ATP at the catalytic center (Fig. 6, and Hountondji et al., 1985; Mechulam et al., 1991; Kalogerakos et al., 1994).

Fragment [402–465] is located in the C-terminal helical domain of the M547 3D structure, about 75 Å away from the active site crevice. Lys-465 establishes an interaction with the wobble cytidine C34 base of the anticodon of tRNA$_f^{Met}$ (Fig. 6, and Leon & Schulman, 1987). Lys-465 also lies close to Trp-461, a crucial residue in the recognition of the anticodon of tRNA$_f^{Met}$ (Fig. 6, and Meinnel et al., 1991). In affinity labeling studies by Leon and

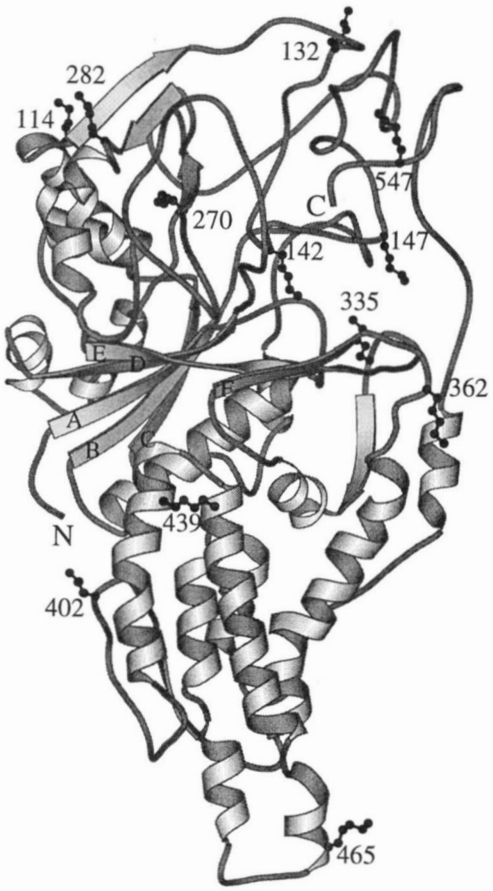

Fig. 6. Ribbon diagram (prepared using the program MOLSCRIPT of Kraulis, 1991) of the available structure of monomeric trypsin-modified MetRS. The lysyl residues identified in this work are represented in ball and stick. With courtesy of Dr. S. Brunie.

Schulman (1987) and Valenzuela and Schulman (1986), lysines-402 and -439 were shown to correspond to the binding sites for the D-loop and the extra arm of $tRNA^{Met}$, respectively.

The carboxyl-terminal Lys-547 of M547 was also found covalently bound to a methionyl residue. Even though the C-terminal fragment of the enzyme is not directly involved in catalysis, it represents a protruding tail involved in the binding and the guiding of the 3′-acceptor end of tRNA toward the active site crevice (Mellot et al., 1989; Kim et al., 1993; Gale et al., 1996).

Because a large fraction of the methionylated lysyl residues of M547 coincide with residues in the active site of the synthetase (Fig. 6), it is tempting to propose that once activated by ATP, a part of the methionine molecules immediately react with the closest lysyl residues. However, the following observations suggest that the donor methionyl–adenylate might equally modify other enzyme molecules or neighboring acceptor protein molecules, by bimolecular contact or through diffusion in the solvent. First, the predominantly labeled Lys-465 is located in the C-terminal helical domain of the M547 3D structure, 75 Å apart from the active site crevice where methionyl–adenylate formation takes place (Fig. 6). Second, once produced by M547, the methionyl–adenylate is capable of attaching to other added proteins, such as bovine serum albumin (BSA), LysRS, or ValRS. In addition, [^{14}C]Met incorporation into BSA increased in parallel with the amount of BSA in the incubation mixture, in agreement with the demonstration by Kern et al. (1985) that aspartyl–adenylate diffuses out of its site of formation on AspRS, prior to aspartylation of acceptor proteins.

In conclusion, the mechanism for in vitro methionylation of M547 may be accounted for by either the in situ covalent reaction of methionyl–adenylate with lysine side chains in the active center, bimolecular contacts between enzyme-bound adenylate, and an acceptor protein, or diffusion, in the solvent of the label toward the lysyl residues of an acceptor protein (including the synthetase itself). The possibility remains, however, that before labeling amino groups in the active center of a synthetase, the reacting adenylate has to exchange from one synthetase molecule to another one.

M547 inactivation varies with the extent of covalent methionine fixation, indicating that among the labeled lysyl residues, some are critical for enzyme activity. For example, the residual activities of [^{32}P]PP_i–ATP exchange and $tRNA^{Met}$ aminoacylation of 1 or 2 μM M547 having incorporated 7.0 ± 0.5 mol of [^{14}C]L-Met/mol enzyme were 20–25% and less than 10% of that of the intact enzyme, respectively. In the same conditions, 10 μM M547 having incorporated only 5.0 mol of [^{14}C]L-Met/mol enzyme retained 60 and 25% of the initial [32P]PP_i–ATP exchange and $tRNA^{Met}$ aminoacylation activities, respectively. Covalent binding of methionine to either of the critical lysines 142, 147, 270, 282, 335, or 465 of M547 can obviously account for the inhibition of enzyme activity, either by steric hindrance or by the shielding of a cationic residue involved in the productive binding of the anionic substrates, ATP and tRNA.

The increased sensitivity of the tRNA aminoacylation activity compared to the isotopic exchange one can be easily explained by the fact that the predominantly labeled Lys-465, which is situated apart from the catalytic center, as discussed above, is not likely to be involved in the ATP–PP_i exchange reaction. In turn, the shielding of Lys-465 is likely to prevent the correct positioning of the anticodon of $tRNA^{Met}$ and, consequently, to specifically affect tRNA aminoacylation activity.

It is expected that the methionylation reaction proceeds through nucleophilic attack of the carbonyl group of methionyl-adenylate by any nucleophile, so that, besides the ϵ-NH_2 of lysine residues, the sulfhydryl group of cysteine, as well as the hydroxyl group of tyrosine, serine or threonine might form an ester bond with the activated methionine. Actually, the following observations indicate that only lysyl residues of M547 were covalently labeled by methionine: (a) the methionylated peptides identified (Table 2) systematically contained each at least one internal lysine that would have been cleaved by trypsin (as it is in the control unlabeled M547 sample followed in parallel), if its ϵ-NH_2 group had not reacted with an activated methionine; (b) in addition, these labeled peptides analyzed by MALDI-MS (Table 2) showed each a mass value increased by 133, corresponding to the covalent addition of a single methionyl residue.

The question whether the methionylation reaction occurs and has a functional role in vivo may be raised. In the cases of Bakers' Yeast aspartyl- and phenylalanyl–tRNA synthetases, kinetic studies showed that the rates of tRNA aminoacylation and amino acid-dependent PP_i–ATP exchange activities were not sensitive to covalent modification by the cognate amino acid. In addition, the auto-phenylalanylation of phenylalanyl–tRNA synthetase required the presence of low levels of zinc or cadmium in the assay. The demonstration in the present study that both the $tRNA^{Met}$ aminoacylation and the methionine-dependent PP_i–ATP exchange activities of MetRS are repressed upon covalent binding of methionine,

makes it possible that the methionylation reaction is involved in a down-regulation of the synthetase activity in vivo. However, careful examination by mass spectrometry of a tryptic digest of native MetRS from a rapidly growing *E. coli* culture did not enable us to detect any trace of methionylation. Noteworthy, in vivo, MetRS is likely to be saturated with either esterified or unesterified $tRNA^{Met}$, the bindings of which may reduce the efficiency of the reaction of methionine incorporation.

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